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Boundaries between Chromosomal Domains of X Inactivation and Escape Bind CTCF and Lack CpG Methylation during Early Development

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Summary

Escape from X inactivation results in expression of genes embedded within inactive chromatin, suggesting the existence of boundary elements between domains. We report that the 5′ end of *Jarid1c*, a mouse escape gene adjacent to an inactivated gene, binds CTCF, displays high levels of histone H3 acetylation, and functions as a CTCF-dependent chromatin insulator. CpG island methylation at *Jarid1c* was very low during development and virtually absent at the CTCF sites, signifying that CTCF may influence DNA methylation and chromatin modifications. CTCF binding sites were also present at the 5′ end of two other escape genes, mouse *Eif2s3x* and human *EIF2S3*, each adjacent to an inactivated gene, but not at genes embedded within large escape domains. Thus, CTCF was specifically bound to transition regions, suggesting a role in maintaining both X inactivation and escape domains. Furthermore, the evolution of X chromosome domains appears to be associated with repositioning of chromatin boundary elements.

Introduction

A number of genes on the mammalian X chromosome escape X inactivation (Disteche et al., 2002; Brown and Greally, 2003). Little is known of the mechanisms that protect escape genes from silencing. X inactivation is achieved through a series of steps initiated during female development (Avner and Heard, 2001; Plath et al., 2002). Genes on the inactive X chromosome undergo sequential epigenetic modifications, including histone modifications, chromatin condensation, and late replication (Plath et al., 2002). DNA methylation at the 5′ CpG islands appears to be a late event not required for initiation and propagation of X inactivation (Sado et al., 2004) but necessary for its stable maintenance (Hansen et al., 2000; Sado et al., 2000).

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10% to 20% of human X-linked genes escape inactivation (Carrel et al., 1999), while only a few mouse genes are reported to escape (Disteche, 1995; Disteche et al., 2002). We have shown that, prior to escape, the mouse *Jarid1c* (Jumonji, AT-rich interactive domain 1C) gene, previously known as *Smcx,* is completely or partially silenced on the inactive X chromosome of embryos, suggesting that this initial silencing is not effectively maintained, resulting in reactivation in adults (Carrel et al., 1996; Sheardown et al., 1996; Lingenfelter et al., 1998). In adult tissues, the 5′ CpG island of escape genes is unmethylated (Goodfellow et al., 1988) and the associated histone code is characteristic of active chromatin (Gilbert and Sharp, 1999; Boggs et al., 2002).

The existence of adjacent domains of inactive and active chromatin along the X chromosome (Goldman et al., 1987) is supported by the findings of clusters of escape genes (Miller and Willard, 1998; Tsuchiya and Willard, 2000). These clusters can differ between mammalian species: for example, mouse *Jarid1c* is the only gene that escapes within a domain, whereas human *JARID1C* is part of a large escape domain (Tsuchiya and Willard, 2000; Tsuchiya et al., 2004). The structure of escape domains has not been characterized in terms of potential chromatin boundary elements that may protect them.

Several well-characterized chromatin insulators contain binding sites for the 11-zinc finger protein CTCF (Bell et al., 1999; Bell and Felsenfeld, 2000; Hark et al., 2000; Filippova et al., 2001). CTCF, a widely expressed nuclear protein that binds to diverse DNA sequences through usage of different combinations of its individual zinc fingers, has been implicated in the enhancer-blocking function of chromatin insulators (Lobanenkov et al., 1990; Filippova et al., 1996, 1998; Bell et al., 1999). CTCF binds DNA in a methylation-sensitive manner (Bell and Felsenfeld, 2000; Hark et al., 2000; Filippova et al., 2001) and plays a role in the maintenance of methylation-free zones of imprinting control regions (Pant et al., 2003; Schoenherr et al., 2003; Fedoriw et al., 2004; Szabo et al., 2004).

Here, we report that CTCF binding sites are present at the 5′ ends of mouse *Jarid1c* and *Eif2s3x* (eukaryotic translation initiation factor 2 subunit 3 γ) and of human *EIF2S3*; each of these genes escapes X inactivation and is adjacent to a gene subject to X inactivation. Their 5′ end is also associated with a high level of histone H3 acetylation. In contrast, no CTCF binding sites are present at the 5′ end of two other escape genes, human *JARID1C* and *KIAA0522*, each adjacent to another escape gene. Furthermore, mouse *Jarid1c* 5′ CpG island functions as a CTCF-dependent chromatin insulator and displays very low DNA methylation during development, with virtually none at the CTCF binding sites. These findings suggest that CTCF may prevent the propagation of DNA methylation and associated chromatin modifications within escape domains, resulting in failure to maintain stable silencing.

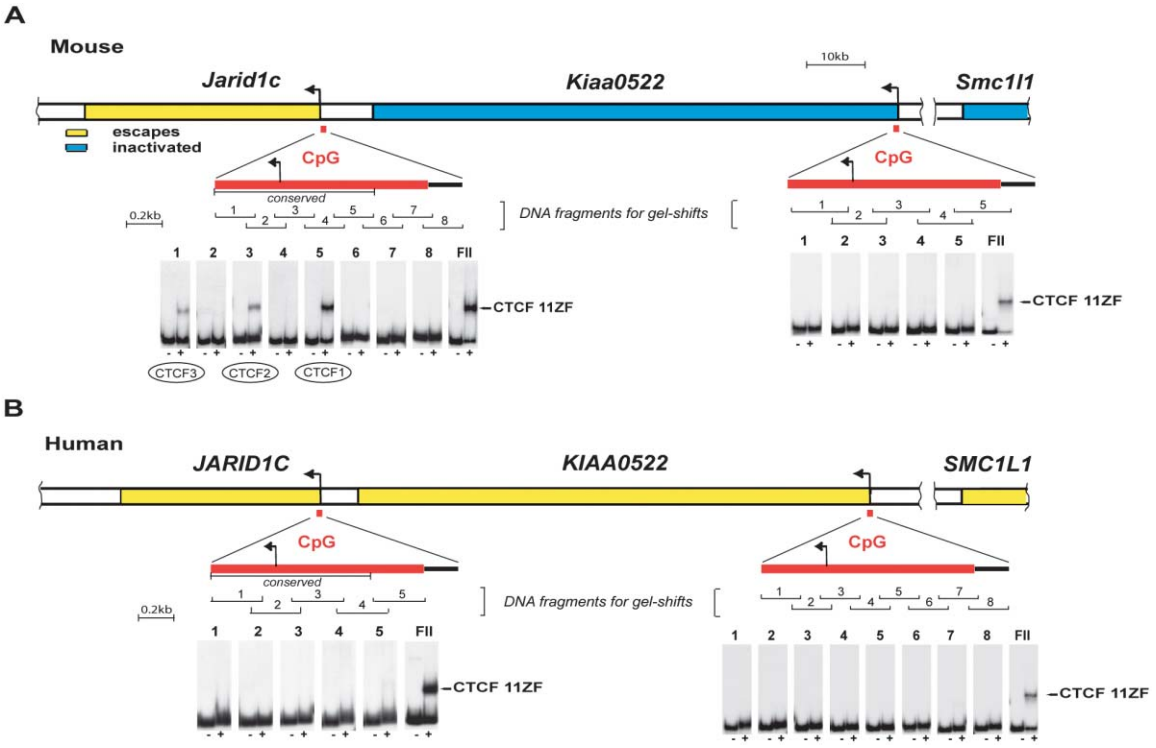

Figure 1. Identification of CTCF Binding Sites at the 5′ End of Mouse *Jarid1c* but Not at Human *JARID1C*

(A) Mouse *Jarid1c* region indicating the inactivation status of genes (blue, subject to X inactivation; yellow, escapes X inactivation), the CpG islands (red), the DNA fragments for gel shift assays, and the region conserved between human and mouse (conserved). Gel shift assays using the 11-zinc finger domain of CTCF (+) or lysate control (−) are shown under the map. A gel shift was observed for *Jarid1c* fragments 1, 3, and 5, corresponding to CTCF binding sites 3, 2, and 1, respectively. No gel shift was observed for *Kiaa0522*. Control positive gel shifts were obtained for the known CTCF binding region of the chicken β-globin gene (FII).

(B) Human *JARID1C* region. Same analysis as in (A). There was no evidence of CTCF binding sites at *JARID1C* and *KIAA0522*.

Results

Escape Domains Differ between Mouse and Human

The 5′ end of *Jarid1c,* a mouse gene known to escape X inactivation, is located about 7 kb away from *Kiaa0522,* a gene subject to X inactivation (Figure 1A; Tsuchiya et al., 2004). The overall genomic organization of these genes is conserved in human, but both human *JARID1C* and *KIAA0522* escape X inactivation within a domain that spans about 285 kb and contains six apparently independent transcripts (Figure 1B; Tsuchiya et al., 2004). Sequence comparison of the 5′ ends of the *Jarid1c*/*JARID1C* genes revealed regions of high homology (77% identity), located up to ~nt −375 relative to the transcription start site (data not shown). *Jarid1c*/*JARID1C* have a dense CpG island and are predicted to encode for transcription factors (http://www.ncbi.nlm.nih.gov). Previous studies using luciferase assays indicate that *Jarid1c* promoter is TATA-less and included in a 2.3 kb region upstream from the transcription start site (Tsuchiya and Willard, 2000).

Another set of domains that differ between mouse and human contain the escape genes *Eif2s3x/EIF2S3* (Ehrmann et al., 1998). At the 3′ end of mouse *Eif2s3x* lies *Zfx* (zinc finger on the X chromosome), a gene subject to X inactivation, whereas *ZFX* escapes, resulting in a larger escape domain in human (Schneider-Gadicke et al., 1989; Adler et al., 1991; Ashworth et al., 1991). *Eif2s3x/EIF2S3* have a dense CpG island and encode translation initiation and elongation factors (http://www.ncbi.nlm.nih.gov). We determined that *6330500C13Rik*, located about 22 kb upstream of *Eif2s3x*, and *AK057298*, about 28 kb upstream of *EIF2S3*, were both subject to X inactivation (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/8/1/31/DC1/). Thus, like mouse *Jarid1c*, both mouse *Eif2s3x* and human *EIF2S3* have their 5′ end located in a transition region between escape and inactivated domains (Figures 2A and 2B).

CTCF Binding Sites Are Located within the 5′ CpG Islands of Escape Genes Adjacent to Inactivated Domains

Gel mobility shift assays to search for CTCF binding sites at the 5′ end of mouse *Jarid1c* were done using overlapping PCR-amplified DNA fragments that spanned about 1300 bp within the CpG island, and the in vitro translated DNA binding domain of CTCF (CTCF 11ZF), which has the same sequence specificity as the full-length protein (Figure 1A; Vostrov and Quitschke, 1997). Three fragments interacted with CTCF: fragments 5 (site 1) and 3 (site 2) showed the strongest interaction,

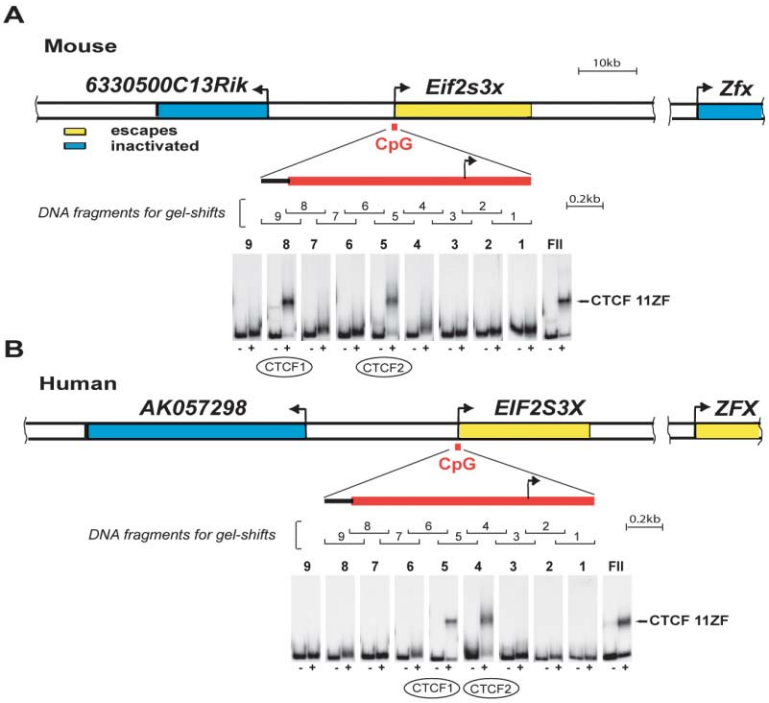

Figure 2. Identification of CTCF Binding Sites at the 5′ Ends of Mouse *Eif2s3x* and Human *EIF2S3*

(A) Mouse *Eif2s3x* region indicating the inactivation status of genes (blue, subject to X inactivation; yellow, escapes X inactivation), the CpG islands (red), and the DNA fragments used for gel shift assays. Gel shift assays using the 11-zinc finger domain of CTCF (+) or lysate control (−) are shown under the map. Gel shifts were observed for fragments 8 (site 1) and 5 (site 2). Control positive gel shifts were obtained for the known CTCF binding region of the chicken β-globin gene (FII).

(B) Human *EIF2S3* region. Same analysis as in (A). Gel shift were observed for fragments 5 (site 1) and 4 (site 2).

whereas fragment 1 (site 3) was weaker and was not characterized further (Figure 1A).

Regions of high DNA sequence similarity between mouse and human *Jarid1c/JARID1C* overlapped the three CTCF sites found in mouse (Supplemental Figure S2A). However, an important difference between the species is that *Jarid1c* is flanked at its 5′ end by *Kiaa0522* and *Smc1l1,* both subject to X inactivation, while *JARID1C*, *KIAA0522*, and *SMC1L1* all escape X inactivation, raising the question of the functional conservation of CTCF binding sites in human. Interestingly, no interaction between CTCF and the 5′ end of human *JARID1C* was found (Figure 1B). Similarly, there was no evidence of CTCF binding at the 5′ CpG islands of either *Kiaa0522* or *KIAA0522* (Figures 1A and 1B).

To determine whether CTCF binding sites were present at the 5′ end of other genes located within a transition region between domains of escape and inactivation, we examined mouse *Eif2s3x* and human *EIF2S3*. Gel mobility shift assays revealed two strong CTCF binding sites at the 5′ CpG islands of both *Eif2s3x* (fragment 8 [site 1] and fragment 5 [site 2]) and *EIF2S3* (fragment 5 [site 1] and fragment 4 [site 2]) (Figures 2A and 2B).

Taken together, our data indicate that CTCF binding sites are not simply associated with genes that escape but appear to be specifically located at transition regions between domains.

Characterization of CTCF Binding Sites

To confirm specific binding of CTCF to *Jarid1c* 5′ CpG island, we used gel mobility shift assays with the in vitro translated full-length protein (in vitro CTCF) and with nuclear extracts from several cell types, including mouse CB3, human K562, and primary human fibroblasts. Specificity of CTCF binding was established using antibodies and competitors (Figure 3A and data not shown). A similar analysis was done for *Eif2s3x* and *EIF2S3*, confirming specific CTCF binding to their 5′ end (Figures 3B and 3C).

Footprinting analysis was used to delineate the CTCF binding sites within the DNA fragments positive for a gel mobility shift. CTCF protected from DNase I digestion about 50 bp on both strands at each *Jarid1c* site, and methylation interference assays identified "contact" guanines whose methylation prevented or reduced CTCF binding (Figure 3A). *Jarid1c* sites 1, 2, and 3 were mapped relative to the transcription start site to nt −376 to −320, nt −111 to −55, and approximately nt 160 to 215, respectively. Each site had 2–3 CpG dinucleotides containing contact guanines. Several contact guanines were not preserved in the corresponding human *JARID1C* sequence, which may explain the lack of CTCF binding (Supplemental Figure S2A). DNA methylation abolished CTCF binding, as shown by gel mobility shift assays on in vitro methylated DNA (Supplemental Figure S3 and data not shown). DNase I footprinting and methylation interference assays (Supplemental Figure S4) were done to map *Eif2s3x* sites to nt −1090 to −1138 (site 1) and nt −563 to −614 (site 2) and *EIF2S3* sites to nt −645 to −697 (site 1) and nt −514 to −565 (site 2), respectively (Figures 3B and 3C). The sequence of CTCF site 2, but not site 1, was partially conserved between species (Supplemental Figure S2A).

Aside from the presence of clusters of contact guanines, comparison of *Jarid1c*, *Eif2s3x*, and *EIF2S3* binding sites showed no evidence of a single consensus sequence and little similarity to previously published CTCF sites (Supplemental Figures S2B and S2C).

In Vivo Binding of CTCF in Transition Regions

Chromatin immunoprecipitation (ChIP) using a monoclonal antibody against CTCF was done on chromatin

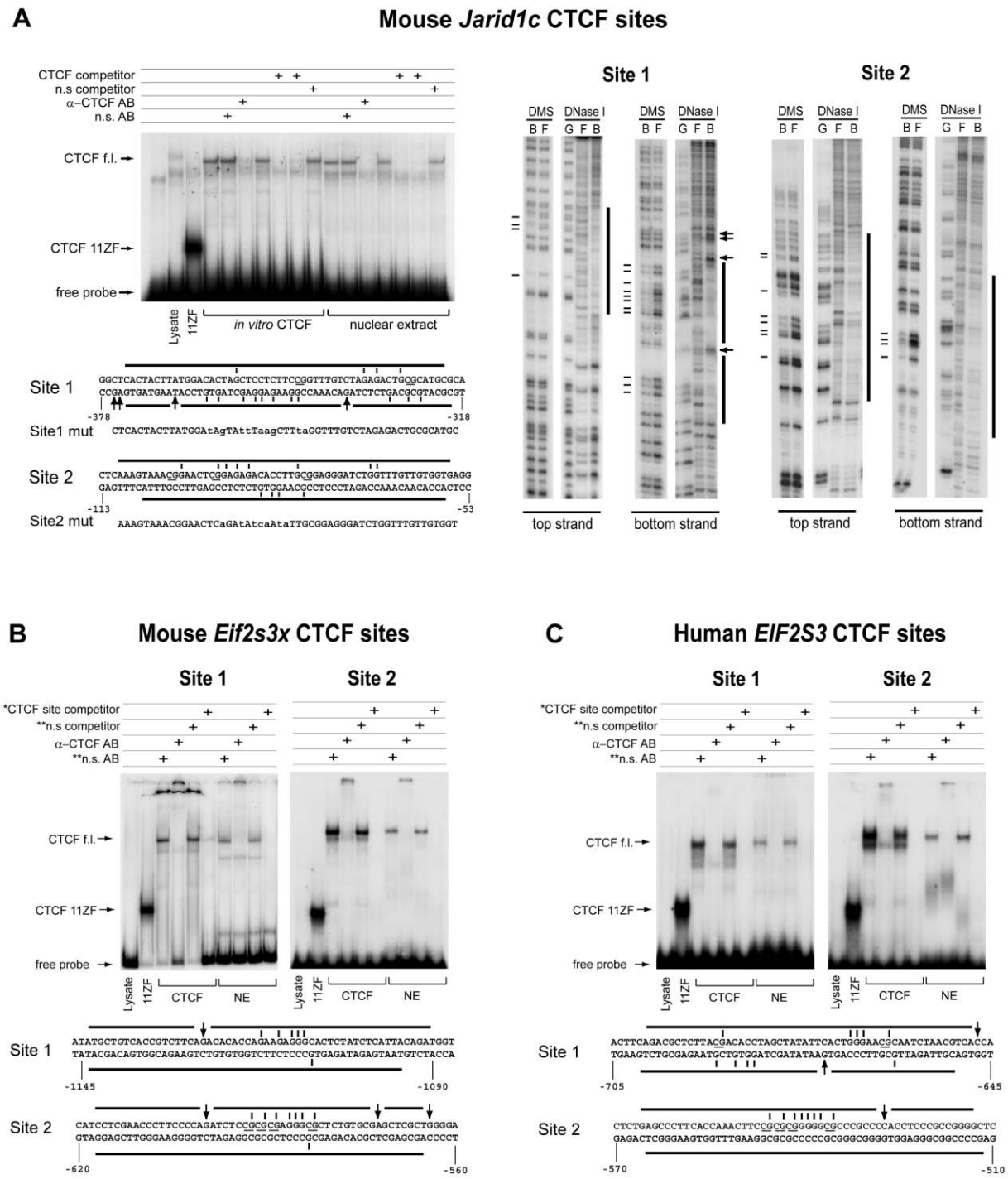

Figure 3. Characterization of the CTCF Binding Sites

(A) Mouse *Jarid1c* CTCF sites. Upper left: Gel shift assays of site 1 show specific interactions with both in vitro translated and endogenous CTCF (nuclear extracts). Addition of either anti-CTCF antibody (α-CTCF AB) or a competitor containing known CTCF sites from the chicken β-globin (FII) or *H19* loci abolished the gel shift. Nonspecific competitor (ns competitor) or antibody (ns AB) did not affect binding. fl, full-length protein; 11ZF, DNA binding domain; arrows, shifted protein-DNA complexes. Right: DNase I footprinting and methylation interference (DMS) analyses of sites 1 and 2. Top and bottom strand indicate strand end-labeled with ^{32}P. Lane B, CTCF bound DNA probes; lane F, free DNA probes; lane G, Maxam-Gilbert sequencing G ladder; horizontal bars, guanines essential for CTCF recognition; vertical bars, regions protected from DNase I digestion; arrows, DNase I-hypersensitive sites induced by CTCF binding. Bottom left: Sequence of sites 1 and 2. Vertical bars, contact guanines; horizontal bars, regions protected from DNase I digestion; arrows, DNase I hypersensitive sites; underlined, CpG dinucleotides; Site1mut, mutation disrupting CTCF site 1; Site2mut, mutation disrupting CTCF site 2.

(B) Mouse *Eif2s3x* CTCF sites. Top: Gel shift assays of sites 1 and 2. Same analysis as in (A) upper left. NE, nuclear extracts. Bottom: Sequence of sites 1 and 2 (same annotation as in [A]), as determined by DNase I footprinting and methylation interference analyses (Supplemental Figure S4).

(C) Human *EIF2S3* CTCF sites. Same analysis as in (B).

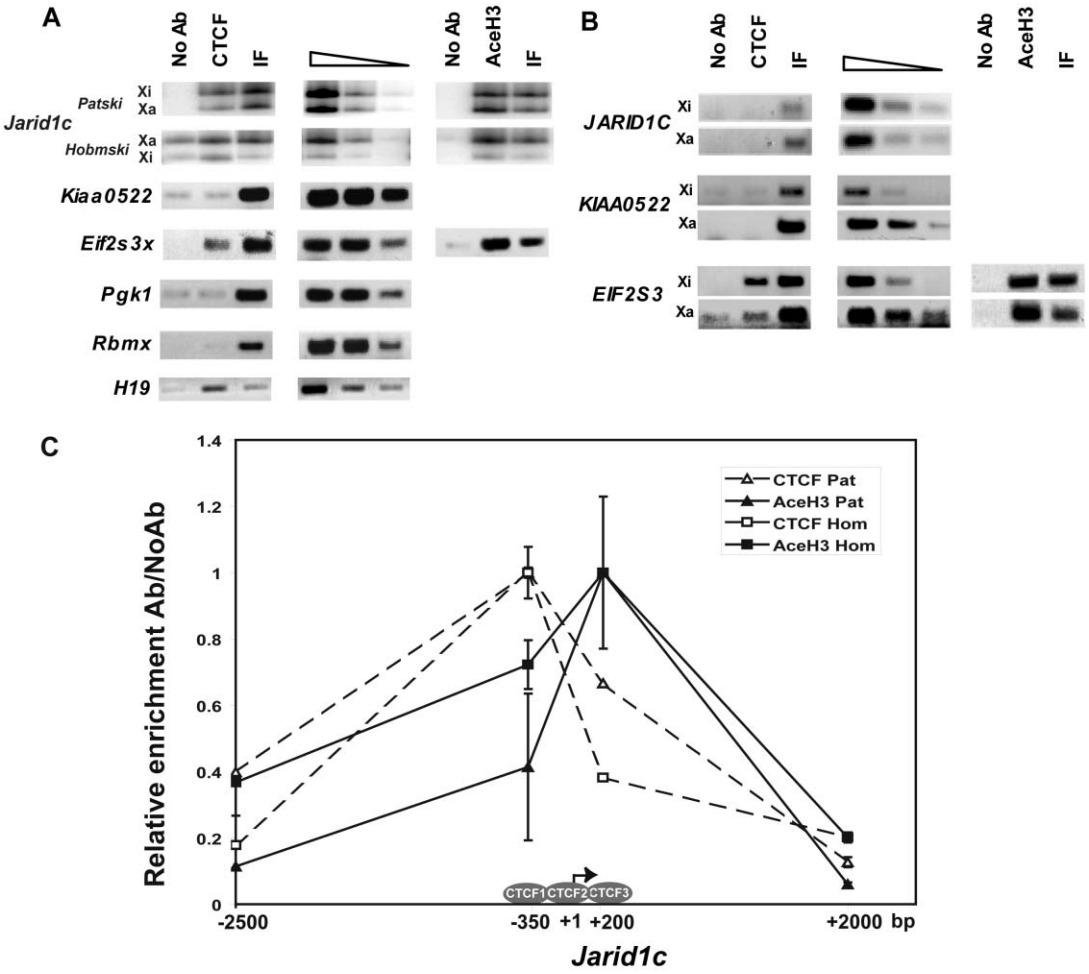

Figure 4. In Vivo CTCF Binding and Histone H3 Acetylation Analyses by ChIP

(A) ChIP for the 5′ ends of mouse *Jarid1c, Kiaa0522, Eif2s3x, Pgk1,* and *Rbmx* using a CTCF antibody and of *Jarid1c* and *Eif2s3x* using an acetylated histone H3 (AceH3) antibody. PCR amplifications of fractions obtained in the absence of antibody (No Ab), in the presence of antibody (CTCF; AceH3), and of the input fraction (IF) are shown for Patski cells, except for *Jarid1c,* also shown for Hobmski cells. Control PCR amplifications of decreasing amounts of IF demonstrate decreased band intensity. *Jarid1c* and *Eif2s3x* and the control *H19* gene, but not the other genes, show enrichment in the CTCF antibody lane; *Jarid1c* and *Eif2s3x* also show enrichment in the AceH3 lane. Xa, active X allele; Xi, inactive X allele.

(B) ChIP for the 5′ ends of human *JARID1C, KIAA0522, EIF2S3* in hybrid cell lines that retains either an inactive X (Xi) or an active X (Xa). PCR amplifications show enrichment in the CTCF antibody lane (CTCF), compared to the no-antibody lane (No Ab) for *EIF2S3*, but not for *JARID1C* and *KIAA0522*. *EIF2S3* also shows enrichment in the acetylated histone H3 antibody lane (AceH3).

(C) Relative enrichment (as a fraction of the highest enrichment for each antibody) for CTCF (dotted line) and acetylated histone H3 (solid line) in the *Jarid1c* region for Patski (Pat) and Hobmski (Hob) cell lines. PCR assays were done at ~nt −2500, ~nt −350 (gel shown in [A]), ~nt 200, and ~nt 2000, from the transcription start site (arrow). Each point represents average enrichment (±standard error) in the antibody fractions. The position of CTCF sites is indicated.

prepared from mouse cell lines with skewed X inactivation of either the *M. spretus* X (Hobmski) or the C57BL/6J X chromosome (Patski) (Bressler et al., 1993; Lingenfelter et al., 1998) and from two hybrid cell lines retaining the human X chromosome, either active or inactive (Lingenfelter et al., 2001). PCR analysis showed enrichment of the products corresponding to the 5′ ends of mouse *Jarid1c* and *Eif2s3x* and human *EIF2S3* in the CTCF ChIP fractions, confirming our in vitro results (Figures 4A and 4B). *Jarid1c* alleles were distinguished by a size polymorphism (10 bp insertion upstream of CTCF site 1) in C57BL6/J, as compared to *M. spretus*. CTCF binding to the inactive X (Xi) appeared to be stronger than that to the active X (Xa), after correcting for the ratios found in the input fraction (Figure 4A). Similarly, a higher enrichment for the 5′ end of human *EIF2S3* was observed for the human inactive X (Xi) versus the active one (Xa) (Figure 4B). However, the apparently stronger CTCF binding to the inactive X may result from different efficiency of DNA/protein cross-linking and/or antibody binding due to different chromatin conformation between the X chromosomes.

Taken together, our data indicated that CTCF was bound in vivo to the 5′ ends of mouse *Jarid1c* and *Eif2s3x* and of human *EIF2S3*. PCR amplifications of the 5′ ends of mouse *Kiaa0522* and human *JARID1C* and

KIAA0522 showed no enrichment in the CTCF antibody fraction, as predicted (Figures 4A and 4B). To further rule out that the presence of CTCF binding sites was simply a common finding among CpG islands of X-linked genes, ChIP was done on two additional genes subject to X inactivation, *Pgk1* (phosphoglycerate kinase 1) and *Rbmx* (RNA binding motif protein, X chromosome). Although these genes possess CpG islands, there was no evidence of in vivo CTCF binding (Figure 4A).

Mouse *Jarid1c* 5′ End Is Associated with a Peak in Acetylated Histone H3 and Functions as an Insulator

ChIP using an antibody against acetylated histone H3 showed a peak of enrichment at *Jarid1c*, around the CTCF sites (Figures 4A and 4C). The presence of a peak in histone H3 acetylation at *Jarid1c* 5′ end is consistent with its role as a chromatin insulator element (West et al., 2002). Enrichment in acetylated histone H3 was also found at the 5′ ends of *Eif2s3x* and *EIF2S3* (Figures 4A and 4B).

To determine whether the 5′ end of *Jarid1c* could contribute insulator activity, we compared the enhancer-blocking activity of this region to the well-known insulator HS4 (hypersensitive site 4 from the chicken β-globin locus), using constructs described previously (Bell et al., 1999; Bell and Felsenfeld, 2000; Hark et al., 2000; Filippova et al., 2001). In this system, sequences inserted between an enhancer and the neomycin gene are assayed for a potential reduction in the number of G418-resistant colonies after transfection of constructs. Insertion of the mouse *Jarid1c* region containing CTCF binding sites 1 and 2 (nt −682 to −25), but not of the corresponding human *JARID1C* region (nt −687 to −25), significantly reduced the number of colonies obtained, compared to the parent vector (Figure 5A). The insulation provided by *Jarid1c* was about half as efficient as that of the HS4 insulator and appeared to be independent of insert orientation. Moreover, the insulator function of *Jarid1c* CpG island was absolutely dependent on CTCF binding: mutations within the recognition core sequence of both sites 1 and 2 (Figure 3C) eliminated binding (data not shown) and resulted in almost complete loss of insulator activity (Figure 5A).

To assay the 5′ end of *Jarid1c* for potential repressor activity and/or insulation from position effect, we inserted it upstream of the neo gene-enhancer cassette in place of the HS4 insulator (Figure 5B). Comparison of constructs, including mouse *Jarid1c*, the mutated *Jarid1c,* and the human *JARID1C* to the HS4 and the insulator-less constructs, showed no decrease in the number of G418-resistant colonies for the 5′ end of mouse *Jarid1c,* which, like the HS4 insulator, did not have inhibitory effect on the neomycin gene (Figure 5B). In fact, there was a significant increase in the number of G418-resistant colonies specifically for *Jarid1c* inserted in the 5′-3′ orientation, indicating that this region increased the likelihood that the reporter gene remained expressed at random genomic sites and suggesting potential insulation from position effects.

Taken together, these data indicate that the CTCF binding sites at the 5′ end of *Jarid1c* play a role in the insulator activity of this region.

DNA Methylation at *Jarid1c* CpG Island Is Very Low in Embryos and Consistently Absent from CTCF Binding Sites

Our previous studies have shown that escape of *Jarid1c* from X inactivation is preceded by silencing during development (Lingenfelter et al., 1998). Silencing by X inactivation is usually accompanied by methylation of the CpG island (Plath et al., 2002). To determine whether *Jarid1c* inactivation and reactivation were associated with methylation changes during development, bisulfite sequencing was done for a region containing a total of 53 CpG sites from nt −570 to 281. Little or no methylation was found at any CpG sites. From embryonic to adult stages, male and female mice showed 90%–100% of molecules being unmethylated at the majority of CpG sites (Figure 6). A single CpG site 24, which displayed slightly higher DNA methylation in 7.5 dpc female embryos as compared to male embryos (13% molecules methylated), became rapidly unmethylated at later stages.

Taken together, our data indicated that DNA methylation was generally very low at the 5′ end of *Jarid1c* and that the partial DNA methylation observed at the single site 24 in early female embryos was rapidly lost during development. In both embryos and adult tissues, our analyses showed that DNA methylation was virtually absent from CpG sites 19–21, 31–33, and 43–47 located within CTCF binding sites 1, 2, and 3, respectively (Figure 6).

Discussion

Chromatin insulators protect domains of the genome from being activated or silenced due to either inappropriate action of outside enhancers/silencers or proximity to inactive condensed chromatin. In the context of X inactivation, such insulators could help maintain expression of specific genes within domains on the inactive X chromosome, either by protecting escape genes from silencing or by protecting inactivated genes from activation. To our knowledge, this is the first report on the characterization of boundary elements between adjacent domains of inactive and active chromatin along the inactive X chromosome. We have determined that the chromatin insulator protein CTCF binds to the 5′ end of three genes, mouse *Jarid1c* and *Eif2s3x* and human *EIF2S3,* all three located in transition regions between domains. In contrast, other genes, either subject to X inactivation or escape genes not at a transition region, did not show association with CTCF. In particular, we have demonstrated that CTCF binding sites are absent at human *JARID1C*, which, in contrast to the mouse gene, is part of a large escape domain (Tsuchiya and Willard, 2000; Tsuchiya et al., 2004). CTCF binding sites at the 5′ end of mouse *Jarid1c* could elicit an insulator activity in enhancer blocking and protection from position effects assays, but the corresponding region in human *JARID1C* could not. These findings suggest that escape is probably not regulated at the level of individual genes, but rather at the level of the chromatin domains. Finally, we have shown that CpG methylation is very low at *Jarid1c* 5′ end throughout development, suggesting that CTCF may interfere with the establishment of methylation.

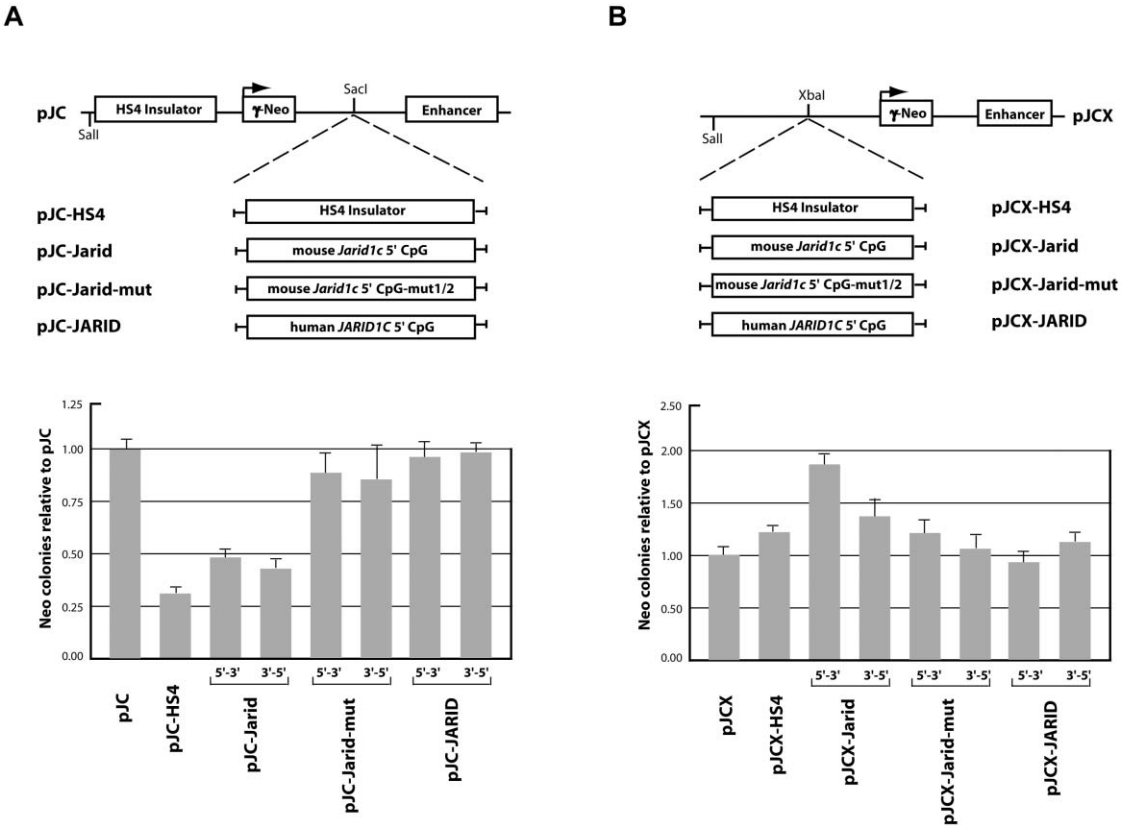

Figure 5. Insulator Activity of the Mouse *Jarid1c* 5′ End Dependent on CTCF Binding

(A) Enhancer-blocking assays: mouse *Jarid1c* CpG island, the same with mutated CTCF sites (Figure 3A), human *JARID1C* CpG island, and a control known insulator (HS4 from the chicken β-globin gene) were cloned into the pJC vector at the SacI site between the HS2 enhancer and the neo gene driven by the γ-globin promoter, with an adjacent insulator element HS4 from the chicken β-globin gene. Colony assays of K562 cells selected in G418 (histogram below) demonstrate that mouse *Jarid1c* 5′ end (pJC-Jarid) but not human *JARID1C* functions as an insulator. Mutation of both *Jarid1c* CTCF sites (pJC-Jarid-mut) almost completely eliminates insulator activity. Fragments were tested in forward (5′-3′) and in reverse (3′-5′) orientations. pJC-Jarid resulted in a reduction to about 40% (3′-5′) to 50% (5′-3′) colonies, compared to the pJC vector. The positive control pJC-HS4 containing the chicken HS4 insulator showed a reduction to about 30% colonies. Results shown as an average (with standard error) of at least six independent transfections performed in triplicate.

(B) Repressor activity and protection from position effects assays: the same fragments described above (A) were cloned outside the promoter-enhancer cassette of the pJCX vector at the XbaI site. Colony assays of K562 cells selected in G418 (histogram below) demonstrate that mouse *Jarid1c* 5′ end (pJCX-Jarid) does not have repressor activity on the neo gene promoter and provides potential protection from position effects, especially in the 5′-3′ orientation. pJCX-Jarid (5′-3′) resulted in an approximately 80% increase in the number of colonies, compared to the control pJCX vector. This was the case neither for the human *JARID1C* construct (pJCX-JARID) nor for the construct with mutated CTCF sites (pJCX-Jarid-mut). The positive control pJCX-HS4 containing the chicken HS4 insulator showed a 20% increase in colonies. Results shown as an average (with standard error) of at least four independent transfections performed in triplicate.

Several well-characterized chromatin insulators contain CTCF binding sites (Bell et al., 1999; Bell and Felsenfeld, 2000; Hark et al., 2000; Filippova et al., 2001; Magdinier et al., 2004) and are often associated with a peak of acetylation at histone H3 (Litt et al., 2001), as we have observed for *Jarid1c* and *Eif2s3x*/*EIF2S3*. CTCF binding sites sensitive to DNA methylation have also been located within the imprinting control region of the *H19*/*Igf2* locus (Bell and Felsenfeld, 2000; Hark et al., 2000) and near the 3′ end of *Xist*, where they may play a role in X inactivation choice (Chao et al., 2002). In the case of the HS4 β-globin gene insulator, CTCF has been specifically implicated in the enhancer-blocking activity of the insulator but not in protection from position effect (Recillas-Targa et al., 2002). However, CTCF can provide protection from position effect in a yeast assay, where it can block the spreading of repressive telomeric chromatin (Defossez and Gilson, 2002). A recent study suggests that the chromatin insulator activity of CTCF may be mediated by the formation of loops tethered to the nucleolus through nucleophosmin binding (Yusufzai et al., 2004). Moreover, dimerization may mediate interactions between CTCF-DNA complexes that could provide anchor points for the formation of chromatin loops (Pant et al., 2004). In the case of the X chromosome, inactivated and escape domains could be located in separate chromatin loops, potentially anchored by CTCF and other insulator elements. Our preliminary analysis of the region located 3′ to mouse *Jarid1c* showed the presence of CTCF binding sites at a CpG island positioned at the 3′ end of *DXBwg1396e*, a gene subject to X inactivation, which mapped about 63 kb from the 3′ end of *Jarid1c*.

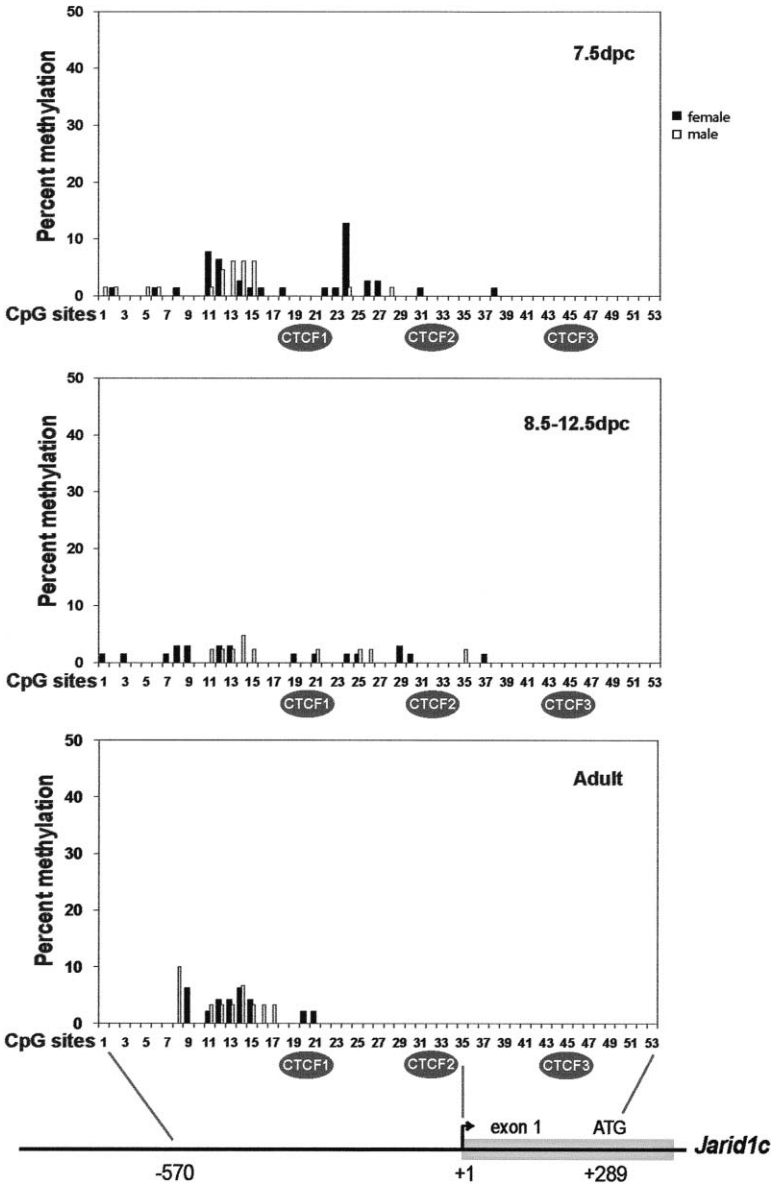

Figure 6. DNA Methylation at the 5′ CpG Island of *Jarid1c* in Embryos and Adult Tissues

DNA methylation was assayed by bisulfite sequencing at 53 CpG sites (x axis of histograms) covering the CpG island of *Jarid1c* from nt −570 to the beginning of the coding sequence within exon 1 (nt 289) and including the CTCF binding sites. The percent of methylated CpG sites (y axis of histograms) is shown for 7.5 dpc embryos (65 clones from 4 males and 79 clones from 4 females), 8.5–12.5 dpc embryos (42 clones from 7 males and 59 clones from 10 females), and adult tissues (30 clones from 3 males and 24 clones from 3 females). Embryos and tissues from males are in white and females are in black. CTCF binding sites 1, 2, and 3 remain essentially unmethylated.

This suggests the presence of a potential boundary on the other side of *Jarid1c,* which could mediate the formation of a chromatin loop (Supplemental Figure S5A). X chromosome-wide screening for CTCF binding sites will help further delineate chromatin domains. Others and we have observed a focus of CTCF antibody staining within the Barr body (Supplemental Figure S5B; Chadwick and Willard, 2003). It will be interesting to determine whether the binding sites we have identified here localize to this focal region.

CTCF is also known as a transcription repressor or activator of some genes (Filippova et al., 1996; Vostrov and Quitschke, 1997). It could be argued that CTCF directly regulates *Jarid1c* and/or *Eif2s3x/EIF2S3* expression. However, our findings of CTCF sites at these three different genetic loci, all located at transitions regions between domains of inactivation and escape, suggest a common function for these sites in insulating domains. Additional evidence against a role for CTCF as a transcription factor for all three escape genes includes: (1) as we have shown, the 5′ end of *Jarid1c* does not function as a repressor; and (2) despite the difference in CTCF binding at their 5′ end, mouse *Jarid1c* and human *JARID1C* are highly conserved and show similar expression patterns (Agulnik et al., 1994; Wu et al., 1994a, 1994b).

While the CTCF binding sites that we have characterized display some similarity to previously reported sites based on the presence of a GC-rich core sequence, we could not derive a definitive consensus sequence. The presence of additional contact guanines outside of the previously defined core sequence suggests that this core is not sufficient for binding. Furthermore, CTCF does not bind to the 5′ end of human *JARID1C,* despite the DNA sequence similarity between mouse and human. This confirms the difficulty in predicting binding sites based on DNA sequence analysis alone and reflects the ability of CTCF to bind diverse DNA sequences by different combinations of its individual zinc fingers (Filippova et al., 1996). CTCF binding sites tend to be

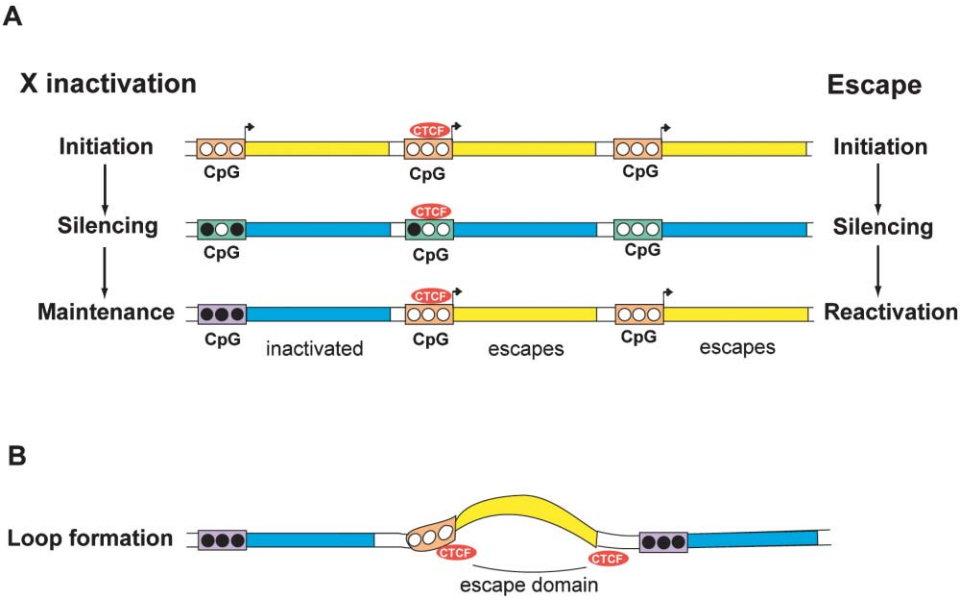

Figure 7. Model for the Role of CTCF Binding and DNA Methylation in a Hypothetical Domain of Escape from X Inactivation

(A) Prior to X inactivation in embryos, both a gene that escapes and a gene that will become stably inactivated are expressed (yellow). At the 5′ end of the genes, the histone code is presumably characteristic of active chromatin (orange) and the CpG island is presumably unmethylated (white dots). Following initiation and spreading of X inactivation, early histone modifications (green) would take place and genes on the inactive X become silenced (blue). At a later stage (maintenance), genes that become stably inactivated undergo additional changes including DNA methylation (black dots) at their CpG island and further histone modifications (purple). However, escape genes would be protected from the propagation and establishment of these latter changes by the presence of chromatin insulator elements such as CTCF, located at transition regions between escape and inactivated domains. Lack of DNA methylation and associated chromatin modifications would result in progressive reactivation and escape.

(B) CTCF binding at both ends of an escape domain may isolate such domains within a separate chromatin loop.

found in clusters, suggesting cooperative interactions (Pant et al., 2004). We have found three adjacent sites at the 5′ end of *Jarid1c* and two at the 5′ end of *Eif2s3x* and of *EIF2S3*. In the case of *Jarid1c*, site 3 has a low-affinity binding to CTCF; such a site may still play an important role in chromatin insulation by cooperation with the stronger sites, as shown by mutation of a weak site at the *Igf2/H19* cluster (Pant et al., 2004).

In addition to CTCF, other elements are most likely needed to mediate the insulation of chromatin domains (Ohlsson et al., 2001; Hori et al., 2002; West et al., 2002). Such elements include matrix attachment regions (MARs) (Mirkovitch et al., 1984). However, a reporter transgene construct flanked by MARs and inserted on the X chromosome was not protected from the spreading of X inactivation and the establishment of CpG methylation (Chong et al., 2002). In the case of *Jarid1c* and *Eif2s3x/EIF2S3*, additional chromatin boundary elements may be located elsewhere within the genes and/or at their 5′ or 3′ end. Several Sp1 consensus binding sites have been previously reported at *Jarid1c* CpG island (Tsuchiya and Willard, 2000). Using the Match program (http://www.generegulation.de), we have also identified a potential binding site for the YY1 transcription factor, downstream from CTCF site 2 and very close to *Jarid1c* transcription start. YY1 can play the dual role of a repressor/activator (Weill et al., 2003) and may also be involved in chromatin insulation (Kim et al., 2003). However, unlike the CTCF binding sites reported here to be specifically associated with mouse *Jarid1c*, the potential YY1 binding site was also present at the 5′ end of human *JARID1C* and no YY1 sites were predicted at the 5′ ends of *Eif2s3x/EIF2S3*.

In the context of X inactivation, which is associated with CpG island methylation, a role for CTCF in escape suggests potential interference with this methylation process. CTCF binding to a specific DNA sequence is closely linked to CpG methylation, with binding both depending on and regulating methylation. CTCF plays a critical role in the maintenance of the methylation-free status of the maternal imprinting control region of the *Igf2/H19* locus (Pant et al., 2003, 2004; Schoenherr et al., 2003; Fedoriw et al., 2004; Szabo et al., 2004). While initiation and propagation of X inactivation take place in the absence of DNA methylation (Sado et al., 2004), CpG island methylation occurs later and is necessary for the maintenance of stable silencing. Indeed, reactivation of X-linked genes is prevalent in DNA methyltransferase mutants such as patients with ICF syndrome (immunodeficiency, centromeric instability, and facial abnormalities) caused by mutations in *DNMT3B* (Hansen et al., 2000) and mice deficient in *Dnmt1* (Sado et al., 2000).

Our current study reports the detailed analysis of CpG island methylation changes in an escape gene during development. Previous studies have shown that escape genes lack CpG island methylation in adult tissues (Goodfellow et al., 1988; Jegalian and Page, 1998). DNA methylation at *Jarid1c* CpG island was very low from 7.5 dpc onward and thus cannot account for the initial silencing of the gene (Lingenfelter et al., 1998). DNA methylation at *Jarid1c* may actually be inhibited during

development, as outlined in our model (Figure 7A). Following this model, insulation of escape genes from adjacent inactive chromatin could be mediated by interactions between CTCF and the establishment and/or cooperative spreading of DNA methylation (Figure 7A). This is supported by the near-complete absence of methylation at the CpG dinucleotides contained within *Jarid1c* CTCF binding sites even during development. In human, *JARID1C* CpG island is also unmethylated (Jegalian and Page, 1998), and yet CTCF does not bind to this gene, suggesting that protection from methylation and stable silencing results from CTCF binding to transition regions between domains, and not to individual escape genes. Presence of both 5′ and 3′ boundary elements flanking an escape domain may mediate the formation of a chromatin loop positioning escape genes in a separate nuclear compartment (Figure 7B).

In addition to remaining unmethylated at their CpG island, escape genes also lack histone modifications that characterize stably silenced genes (Gilbert and Sharp, 1999; Boggs et al., 2002). Escape genes may be marked in embryogenesis by histone modifications that differ from those of genes destined to be silenced (Rougeulle et al., 2003). It will be important to follow histone modifications in escape genes during development. *Jarid1c* initial and unstable silencing could be due to early chromatin modifications without DNA methylation, leading to progressive reactivation of the gene during development (Figure 7A).

It is interesting that the size of escape domains differs between human and mouse, which may be associated with repositioning of chromatin boundary elements that protect domains. In addition to insulating escape genes from silencing by X inactivation, insulator elements could possibly protect inactivated genes from reactivation due to their proximity to escape domains. CTCF has been shown to protect genes from being turned on by nearby enhancer elements (Bell et al., 1999; West et al., 2002; Zhao and Dean, 2004). In that case, the absence of CTCF binding sites at *JARID1C* would lead to the much larger domain of escape in human. The construction of mouse models with binding site mutations will help understand the different functions of CTCF at escape domains on the inactive X chromosome.

Experimental Procedures

Tissues, Cell Lines, and X Inactivation Analyses

Embryos were collected from C57BL/6J mice by dissection at 7.5, 8.5, 9.5, 10.5, 12.5, and 18.5 days postcoitum (dpc). Adult tissues included kidney, liver, and lung. Mouse cell lines in which either the *M. spretus* X (Hobmski) or the C57BL/6J X chromosome (Patski) was always inactive and Chinese hamster × human hybrid cell lines retaining either an active (Y.162.11C) or an inactive (X8.6T2H1, 8121-TGRD, and THX88) human X chromosome were previously characterized (Bressler et al., 1993; Lingenfelter et al., 1998, 2001). The X inactivation status of mouse and human genes was determined as previously described (Lingenfelter et al., 1998; Tsuchiya et al., 2004). Primers are listed in Supplemental Table S1.

Gel Mobility Shift, DNase I Footprinting, and Methylation Interference Assays and In Vitro CpG Methylation Analyses

Characterization of CTCF binding sites by gel shift, DNase I footprinting, and DMS-methylation interference assays and by in vitro CpG methylation analyses was done as described (Filippova et al., 2001). Primers are listed in Supplemental Table S1.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was done following a method previously described (Litt et al., 2001). After formaldehyde treatment to cross-link DNA and proteins, immunoprecipitation reactions were set up using sonicated chromatin (200 bp to 1 kb size range) and antibodies against CTCF (monoclonal antibody, BD Transduction Laboratories) and acetylated histone H3 (multiple acetylated residues, Upstate Biotechnology). PCR (30 cycles) was done using primers listed in Supplemental Table S1. A minimum of 2–3 ChIP experiments was done for each antibody. PCR products were examined by polyacrylamide gel electrophoresis and products quantified by phospho-imaging or real-time PCR. Data for the ChIP fractions were normalized to the no-antibody fractions.

Insulator Assays

pJC-Jarid and pJC-JARID were derived by replacing the HS4 insulator at the SacI site by a ~700 bp fragment (in both orientations) from the 5′ end of mouse *Jarid1c* (containing CTCF sites 1 and 2) and human *JARID1C,* respectively. pJCX-Jarid and pJCX-JARID were obtained by replacing the HS4 insulator at the XbaI site of the pJC-HS4 plasmid with the same fragments. Primers used to derive the fragments are listed in Supplemental Table S1. To generate pJC-Jarid-mut, we mutated both CTCF sites 1 and 2 using QuikChange Multi Site-Directed Mutagenesis protocol (Stratagene) and oligonucleotides listed in Supplemental Table S1. Sequencing was done to confirm identity of the clones. K562 cells were transfected with each linearized construct by electroporation and colonies that grew in G418 were counted as described (Filippova et al., 2001).

DNA Methylation Assays

Bisulfite treatment of genomic DNA was done essentially as described (Tremblay et al., 1997). PCR and nested PCR were done using primers designed to include cytosine (in the original sequence) but no CpG dinucleotides (Supplemental Table S1). PCR products cloned in a TopoTA vector (24 to 79 individual DNA clones per stage) were sequenced using the Sequencing Facility in the Department of Biochemistry (University of Washington).

Control samples treated with Sss1, which methylates cytosines within CpG, showed methylation at all sites. 50:50 mixtures of Sss1-treated and -untreated DNA samples resulted in about half the DNA clones methylated (data not shown).

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